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Establishment of an Activated Macrophage Cell Line, A-THP-1, and its Properties

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TOMINAGA, T., SUZUKI, M., SAEKI, H., MATSUNO, S., TACHIBANA, T. and KUDO, T. *Establishment of an Activated Macrophage Cell Line, A-THP-1, and its Properties*. Tohoku J. Exp. Med., 1998, **186** (2), 99-119 —— A new macrophage cell line with activated character and unique morphology was isolated by selecting adherent cells from the human monocytic cell line THP-1. The original THP-1 cells had been cultured for more than 9 years using 25 cm^2 flasks, when cells with a different morphology appeared, adhering to the bottoms of the culture flasks. These were selected by discarding floating nonadherent cells at every subculture. Enrichment of adherent THP-1 cells with long processes proceeded during the cultivation. These adherent THP-1 showed remarkable phenotypic changes, not only morphologically, but also functionally. Namely, increased phagocytic activity, HLA-DR expression and MLR stimulator activity were remarkable. This adherent cell line was designated as activated-THP-1 (A-THP-1), since it demonstrated characteristics of activated macrophages continuously without exogenous stimulation. A cloned A-THP-1 cell line (A-THP-1 C1) also showed the same features and contained about 10% multinucleated giant cells probably caused by cell fusion. This A-THP-1 cell line, the first activated macrophage cell line to be established, provides a good model for understanding of activation mechanisms of macrophages and multinucleation. In this paper, morphological, immunological, and biological characters of this cell line are described. ——— macrophage activation; cell line; THP-1 © 1998 Tohoku University Medical Press

Activated macrophage cells have phagocytotic activity, bactericidal activity and antigen presentation capacity, in addition to secreting monokines (Kornbluth and Edington 1986; Hudson et al. 1988; Najar et al. 1990). In order to obtain activated macrophage cells, addition of various stimulators such as lipopolysac-

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charide (LPS) or γ-interferon (γ-IFN) to resting cells is usually required (Santamaria et al. 1989). In cases of experiments using monocytes, or alveolar macrophage cells, cytokines like γ-IFN, interleukin-4 (IL-4), granulocyte-macrophage colony stimulating factor (GM-CSF) play important roles in activation of macrophages (Grabstein et al. 1986; Crawford et al. 1987; Stuart et al. 1988; Geissler et al. 1989; Hori et al. 1989; Munn and Cheung 1989, 1990; Smith et al. 1990), although underlying mechanisms are not fully understood.

Giant cells, such as osteoclast cells, and Langhans cells in pulmonary tuberculosis are examples of multinucleated macrophages (Nasiell et al. 1972). The mechanism of multinucleus formation has attracted attention for a long time, but so far only an involvement of phorbol esters and cytokines has been documented in polynucleation of monocytes/macrophages (Weinberg et al. 1984; Hassan et al. 1989).

THP-1 cell line, a human monocytic leukemia cell line established by Tsuchiya et al. (1980), has macrophage functions. We have continuously cultured THP-1 cells for more than 9 years using 25 cm^2 flasks and cells with a different morphology appeared at the end of this period, adhering to the bottoms of the culture flask. These were selected by discarding floating nonadherent cells at every subculture (twice a week) and enrichment of adherent THP-1 cells with long processes thereby proceeded. These adherent THP-1 showed remarkable phenotypic changes, not only morphologically, but also functionally. Increased phagocytic activity and HLA-DR expression became apparent.

This adherent cell line, designated as activated THP-1 (A-THP-1), since it has features of activated macrophages without addition of any exogenous stimulator, and a cloned A-THP-1 cell line (A-THP-1 C1) with the same characteristics and a tendency to form many multinucleated giant cells are described here. In this paper, attention is concentrated on morphological and immunological features.

Materials and Methods

Establishment of the A-THP-1 cell line

The parent THP-1 cell line was supplied by Dr. Tsuchiya, Department of Pediatric Oncology, Institute of Development, Aging and Cancer, Tohoku University and cultured for 9 years with RPMI 1640 supplemented with 10% fetal bovine serum (FBS) using 25 cm^2 flask. Subculture was usually performed twice a week, discarding about 4 ml of cell suspension (mostly floating). Flasks for THP-1 cultivation were changed every 2 months. The original THP-1 cells were nonadherent, but a minor population of cells began attaching to the bottoms of the flasks after 9 years of cultivation. These were selected by discarding nonadherent cells vigorously. This active subculture procedure was repeated 40 times, then adherent cells constituted the major population, designated as A-THP-1. A-THP-1 demonstrated stable characteristics for more than 1 year.

In this study, three lines of THP-1 cells were examined. O-THP-1 is original

line that was frozen immediately after the establishment and recultured for this study as a control. R-THP-1 is the regular cell line (nonadherent) maintained without selecting nonadherent cells continuously for more than 9 years. The A-THP-1 cell line demonstrated an activated and adherent character. In the course of cultivation, nonadherent cells usually arose 3-4 days after subculture. When these nonadherent cells were transferred to new flasks, they began to attach very soon. Lot differences of FBS did not influence the characteristics of the A-THP-1 cells.

Cell lines

Human T cell lines (TALL-1, Jurkat, MOLT-4F), and Burkitt's lymphoma (Raji), erythroleukemia (K562), histiocytic leukemia (U937) and lung squamous cell carcinoma (Sq-19) cells were used as controls. African green monkey kidney cell line (VERO) was used for mycoplasma detection.

Antibodies

Following antibodies were employed in this study. Anti-actin (mouse IgM, KMI-3112, Advance, Tokyo), anti-CD1a (B17.20.9, mouse IgG2a, Immunotech, Marseille Cedex, France), anti-CD64 (FcγRI, 32.2, mouse IgG1, Medarex, Annandale, NJ, USA), anti-CD32 (FcγRII, IgG2b, Medarex), anti-CD16 (FcγRIII, MG38, IgG1, Nichirei, Tokyo), anti-Mac1 (TIB128, M1/70.15.11.5.HL, rat IgG2b, hybridoma supernatant), anti-CR4 (anti-CD11c, FK24, IgG1, Nichirei), anti-HLA-DR (OKIa1, IgG2, Ortho, Raritan, NJ, USA), anti-S100 protein (polyclonal rabbit antibody, MBL, Nagoya), anti-EBV-VCA (KM105, mouse IgG2a, produced by Kudo) were first antibodies, and FITC conjugated goat anti-mouse IgG (H+L) (EY lab, San Mateo, CA, USA), FITC conjugated goat anti-rabbit IgG (MBL, Nagoya), and FITC conjugated goat anti-rat IgG Fab (Cappel, Costa Mesa, CA, USA) were applied as second antibodies. Flow cytometry was carried out using these antibodies diluted with phosphate buffered saline (PBS) plus 10% host inactivated normal human serum, and 0.1% NaN_3 by Epics V (Coulter Immunology, Hialeah, FL, USA).

Detection of mycoplasma

The first method employed (Chen 1977) was as follows. Culture supernatant from A-THP-1 cells was added to cultures of VERO cells in order to transfer any mycoplasma to VERO cells (indicator cells), then the fixed VERO cells were stained with Hoechst 33258. Secondly, a Gen-probe kit (Gen-probe Inc., San Diego, CA, USA) was used for detecting mycoplasma contamination (Hay et al. 1989). This method employs ^{3}H-labeled mycoplasma DNA probe, which hybridizes rRNA in the culture supernatant of test cells.

Cloning by limiting dilution

A-THP-1 cells were cloned by limiting dilution method, using 30 Gy irradiated R-THP-1 cells as feeder cells. Briefly, two or three A-THP-1 cells and x-irradiated R-THP-1 cells (2×10^4) were distributed to each well of a 96-well microplate and cultured. This limiting dilution method was repeated. The cloned cell line was designated as A-THP-1 C1.

Results

Light microscopic observation

Both O-THP-1 and R-THP-1 cells showed round morphology with floating character, and small cluster formation was noted partially. In contrast to O-THP-1 and R-THP-1 cells, A-THP-1 cells (Fig. 1) were mostly adherent and demonstrated long processes (1 to several per cell) ranging from 30 to 200 μm in length. Among the adherent cells, giant cells (5–6 times larger than mononuclear A-THP-1 cells) were observed. During the course of cultivation using the same flasks unchanged, the populations of giant cells with long processes and multinuclei gradually increased (Fig. 1). Long processes tended to form networks between neighboring giant cells. May-Giemsa staining of A-THP-1 cells demonstrated that 11.8% were multinucleated giant cells which had as many as 20 nuclei (Fig. 2). Among the mononuclear A-THP-1 cells, many dividing cells were noted, but multinucleated cells appeared to be nondividing.

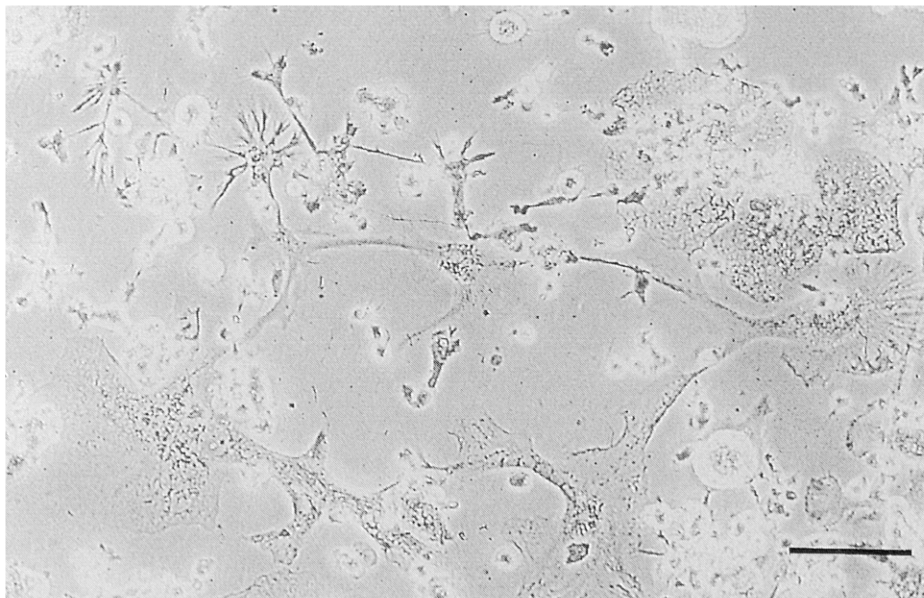

Fig. 1. Micrograph of A-THP-1 cells cultured in the same flask for more than 2 months viewed under a phase contrast microscope. A network of processes from giant cells is apparent. The proportion of giant cells increased during the course of cultivation. Scale bar, 100 μm.

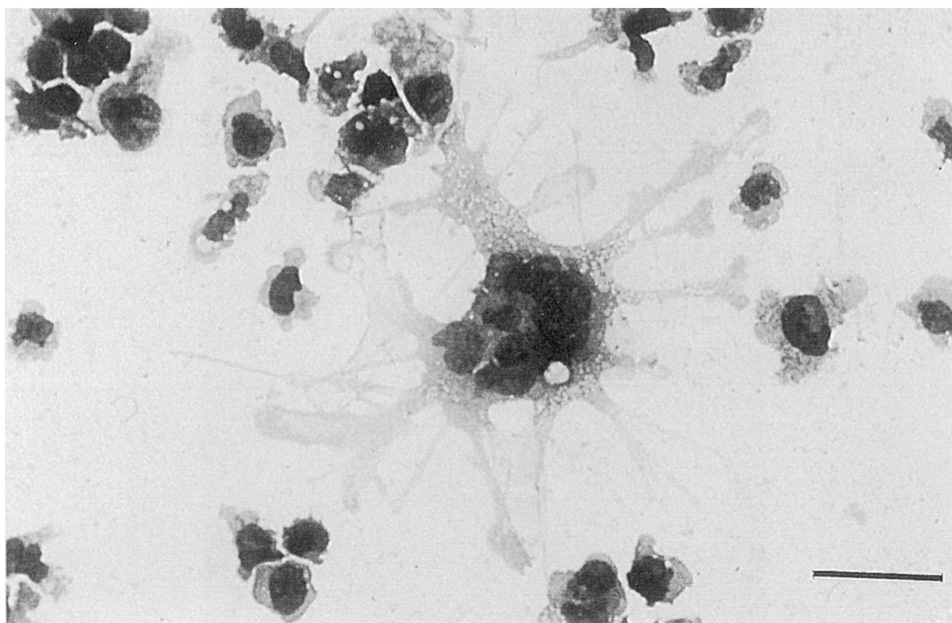

Fig. 2. Micrograph of A-THP-1 cells with May-Giemsa staining ($\times 400$). The cells were cultured on a slide glass, dried, fixed and stained. They consisted of about 90% of mononuclear cells and 10% of multinucleated giant cells. In some cases, 20 nuclei were noted in one giant cell. The processes extending to all directions, ranged in length to about 200 μm. Scale bar, 50 μm.

Electronmicroscopic observation

When O-THP-1 cells were placed on coverglasses, a dome-like morphology without communication to adjacent cells was apparent under a scanning electron-microscope (Fig. 3). On the other hand, A-THP-1 cells showed the irregular morphology with long processes extending in many directions, reaching to neighboring cells (Fig. 4). Though surface of A-THP-1 cells with processes was smooth, they had large volume. Transmission electron microscopy revealed nonadherent cells of all three THP-1 lines to show common characters (Fig. 5), namely, microvilli were prominent with undulating plasma membranes, and irregular nuclei. Though micro-organellae like mitochondria and lysosomes were relatively small in number in R-THP-1 cells, they were more prominent in A-THP-1 cells.

Large A-THP-1 cells with long processes were found to have several nuclei which occupied considerable space within the cells (Fig. 6). Since these nuclei (5-6/cell) had their own nucleoli, we concluded these large cells were multinucleated instead of polymorphonuclear. At high magnification ($\times 3000$) (Fig. 7), mitochondria were numerous especially at the root of processes. Rough endoplasmic reticulum and lysosomes were also apparent along with microtubules (Fig. 8).

Nonspecific esterase staining

All THP-1 cells were stained with α-naphtyl butyrate as reported previously

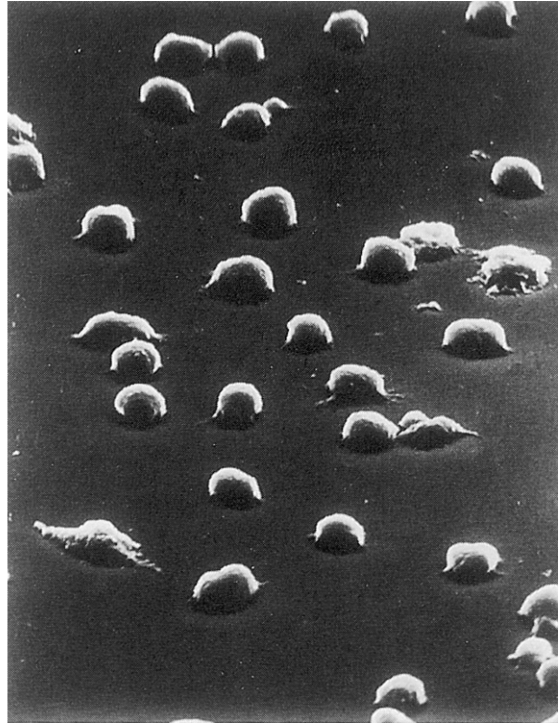

Fig. 3. Micrograph of O-THP-1 cells under a scanning electron microscope ($\times 500$). They showed a round shape, with no communication between adjacent cells. Scale bar, 20 μm.

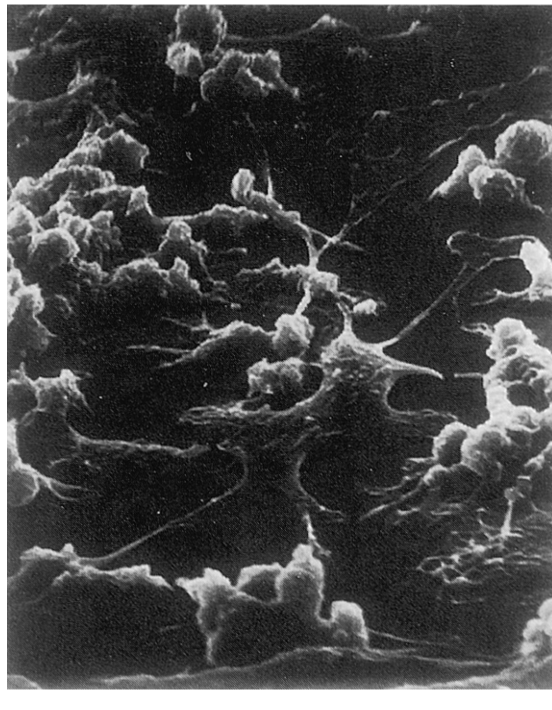

Fig. 4. Micrograph of A-THP-1 cells under a scanning electron microscope ($\times 500$). The cells have long processes extending in all directions and forming a network. Scale bar, 50 μm.

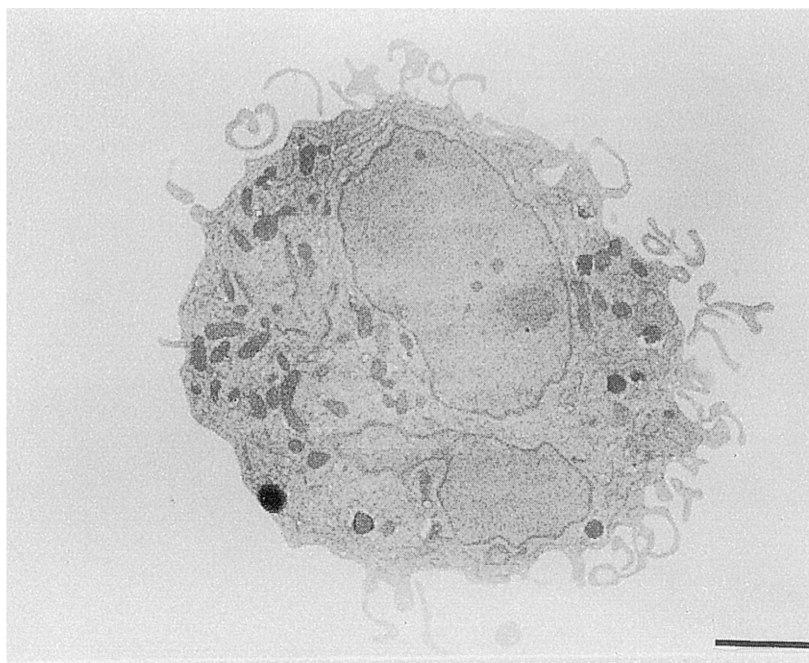

Fig. 5. Micrograph of O-THP-1 cells under a transmission electron microscope ($\times 4000$). The cell has an irregular-shaped nucleus, a moderate number of mitochondria, and endoplasmic reticulum in the cytoplasm. Many microvilli are apparent on the cell surface. Scale bar, 2 μm.

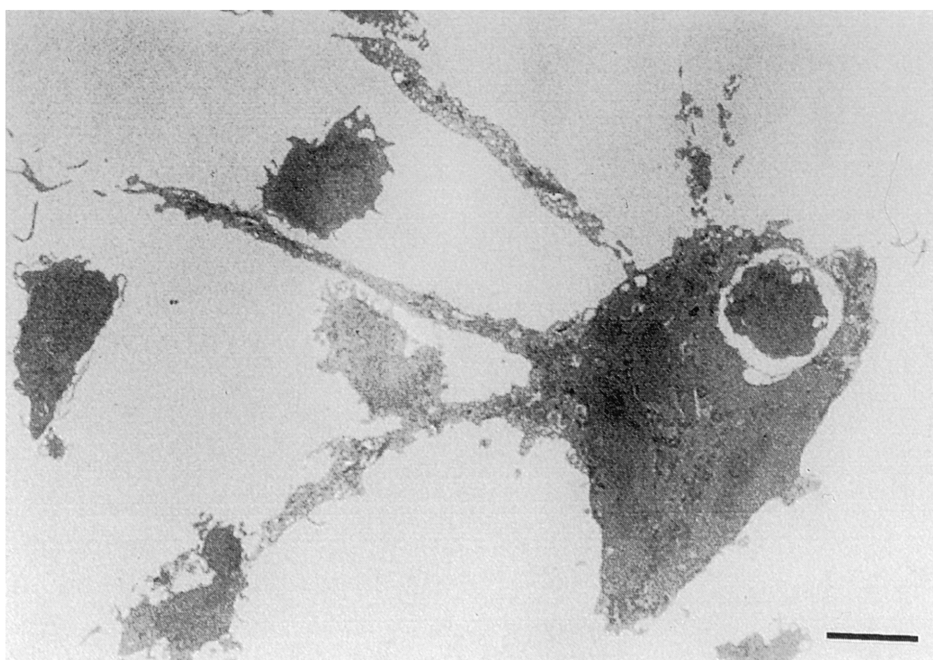

Fig. 6. Electron micrograph on an A-THP-1 cell with long processes, and multiple nuclei ($\times 800$). Note the difference in size from the surrounding mononuclear cells. Scale bar, 20 μm.

(Hoessly et al. 1989; Imai et al. 1989) (Table 1). Staining was blocked by addition of NaF, indicating that these three THP-1 cells were derived from monocytes.

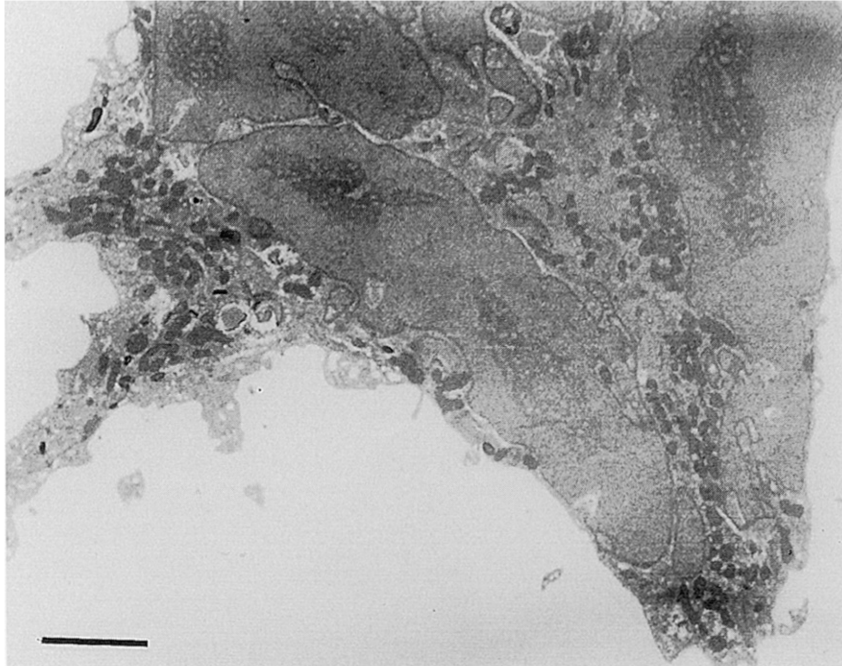

Fig. 7. Higher power view of a giant A-THP-1 cell with long processes ($\times$3000). Microorganelles were abundant at the roots of the processes. Scale bar, 10 μm.

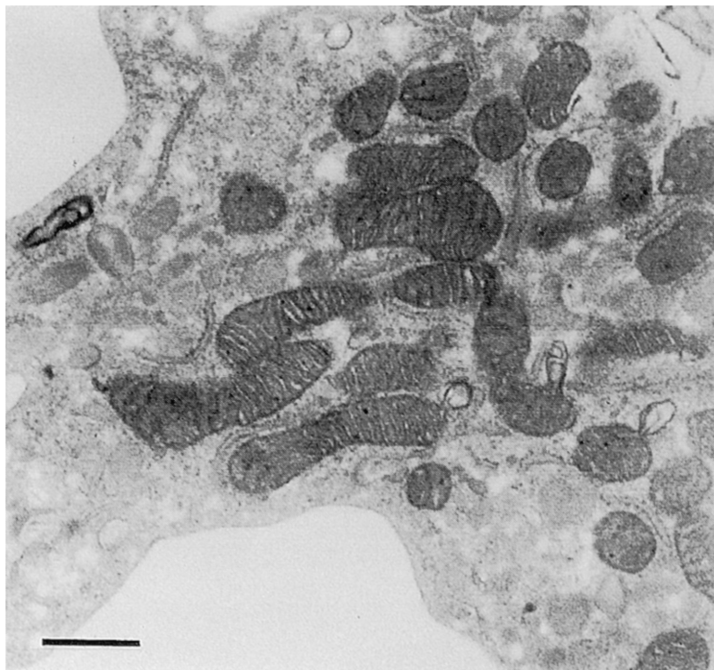

Fig. 8. Detail of cytoplasm ($\times$15 000) of a A-THP-1 cell, illustrating mitochondria, lysosome and rough endoplasmic reticulum at the root of a process. Scale bar, 5 μm.

Chromosomal analysis

All three THP-1 cell lines had chromosomes with human character, with a mode at near 2n. There were some cells with 4n chromosome numbers. Though

TABLE 1. *Nonspecific esterase staining*

Cells	α-naphthyl butyrate	α-naphthyl butyrate+NaF
A-THP-1	+++	−
O-THP-1	+++	−
R-THP-1	+++	−
MOLT-4F	−	−

Test cells smeared on slide glasses were ethanol-fixed, and stained with α-naphthyl butyrate using an esterase staining kit (Muto Chemical, Tokyo). Blocking of nonspecific esterate staining was examined by adding sodium fluoride (NaF).

+++, Strong positive; −, negative staining.

TABLE 2. *Actin and S100 protein expression*

Proteins examined	A-THP-1	O-THP-1	R-THP-1	Jurkat
Actin	+++	+/−	+	NT
S100	++	NT	+/−	+/−

Test cells were smeared, fixed with acetone, and treated with actin-specific or anti-S100 specific antibodies, then FITC conjugated second antibody was added. Staining was observed under a fluorescence microscope (Zeiss standard 18FL). NT, not tested.

about 10% of A-THP-1 cells were multinucleated, giant cells in the A-THP-1 cells, their chromosome distribution was essentially similar to those of O-THP-1 and R-THP-1 cells. This might be because nuclei of multinucleated cells were not fully recovered during the hypotonic treatment and multinucleated cells were nondividing.

LDH isozyme analysis

When LDH isozyme patterns from three THP-1 cell lines were examined using an Authenti kit (Corning Costar, Corning, NY, USA), they showed the same human patterns, indicating a derivation from the same origin.

Actin and S100 protein in the cytoplasm

We examined expression of the cytoskeleton protein actin by indirect immunofluorescence method, since attachment of A-THP-1 cells was remarkably increased (Table 2). Intracytoplasmic actin protein was not detected in O-THP-1 and R-THP-1 cells. However, it could be clearly demonstrated in the processes of A-THP-1 cells. S100 protein was also found in A-THP-1 cells, but neither O-THP-1 nor R-THP-1 cells.

TABLE 3. *E_N rosette formation test*

Cells	E_N rosettes
A-THP-1	6%
Jurkat	5%
TALL-1	45%

E_N was prepared by incubating 1×10^9 SRBC with 0.1 ml neuraminidase (Behringer Mannheim GmbH). E_N rosette tests were performed by incubating test cells with E_N in the presence of FBS on ice in a test tube as reported previously (Kudo and Tachibana 1984a and b). Almost 100% of peripheral blood T cells formed E_N rosettes in this assay. Phagocytosis of E_N was found in most giant A-THP-1 cells.

TABLE 4. *EA rosette formation*

Cells	Exp. 1	Exp. 2	Exp. 3
	Test tube method (%)	Plate method (%)	Plate method (%)
A-THP-1	100	100	100
O-THP-1	30	100	73
R-THP-1	15	100	69
TALL-1	0	NT	NT

SRBCs sensitized with the IgG fraction of rabbit anti-SRBC hemolysin (Kyokuto Pharmaceutical, Tokyo) at subagglutinating concentration were used as indicator cells. In experiment 1, the test tube method was employed. In experiments 2 and 3, the PLL-treated microplate method was used as reported previously (Kudo and Tachibana 1984b; Numasaki et al. 1995).
NT, not tested.

E_N rosette formation

A neuraminidase treated sheep red blood cell (SRBC) (E_N) rosette assay was performed as reported previously (Kudo and Tachibana 1984a and b). None of the three THP-1 cell lines formed E_N rosettes, though phagocytosis of E_N cells by A-THP-1 cells was seen (Table 3).

EA rosette formation

When the EA rosette test for detection of $Fc\gamma$ receptors was performed by the test tube method, A-THP-1 cells were almost 100% positive, while EA rosette positive O-THP-1 and R-THP-1 cells were less than 30% (Table 4). Further

examination using the PLL-treated microplate method (Kudo and Tachibana 1984b; Numasaki et al. 1995), which is more sensitive, all three THP-1 cell lines demonstrated to be 100% positive, but the degree of EA rosette formation in A-THP-1 cells was much stronger than with the other two THP-1 cell lines.

Nitroblue tetrazolium (NBT) reduction test

Reduction activity of THP-1 cells was examined in terms of formazan production in the cytoplasm (Geissler et al. 1989). All THP-1 cells demonstrated formazan particles, but the amounts in A-THP-1 cells were much larger than in

TABLE 5. *NBT reduction test*

Cells	Formazan formation
A-THP-1	+++
O-THP-1	+
R-THP-1	+
TALL-1	−

Test cells were incubated with NBT reagent solution at 37°C for 30 minutes. After washing, blue-violet granular formazan particles in cytoplasm were assessed under a microscope.

TABLE 6. *Phagocytic activity of three THP-1 cells*

Exp. (No.)	Particles tested	Cultured with	Test cells		
			A-THP-1 (%)	O-THP-1 (%)	R-THP-1 (%)
1	SRBC	Inactivated FBS	1-3	0	0
2	SRBC	Inactivated HS	8	0	0
3	SRBC	Fresh HS	27	1	0
4	Latex	Inactivated FBS	100	39	54

In experiment 1, THP-1 test cells and nonsensitized SRBC were incubated at 37°C for 60 minutes in RPMI 1640 plus heat-inactivated 10% FBS. After incubation, nonphagocyted SRBCs were lysed with Gey's lytic solution and then phagocytosis of THP-1 was examined (equivalent to E phagocytosis) by microscopic observation. In experiment 2, THP-1 test cells and nonsensitized SRBCs were incubated at 37°C for 60 minutes in RPMI 1640 plus 10% heat-inactivated human serum (HS). After incubation, nonphagocyted SRBCs were lysed with Gey's lytic solution and then phagocytosis by THP-1 was examined (equivalent to EA phagocytosis). In experiment 3, THP-1 test cells and nonsensitized SRBC were incubated at 37°C for 60 minutes in RPMI 1640 plus 10% fresh HS. After incubation, nonphagocyted SRBCs were lysed with Gey's lytic solution and then phagocytosis by THP-1 was examined (equivalent to EAC phagocytosis). In experiment 4, THP-1 test cells were incubated with latex particles (1.0 μm, Sekisui Chemical, Tokyo) for 60 minutes, and then cells were washed. Latex phagocytosis was observed under a microscope.

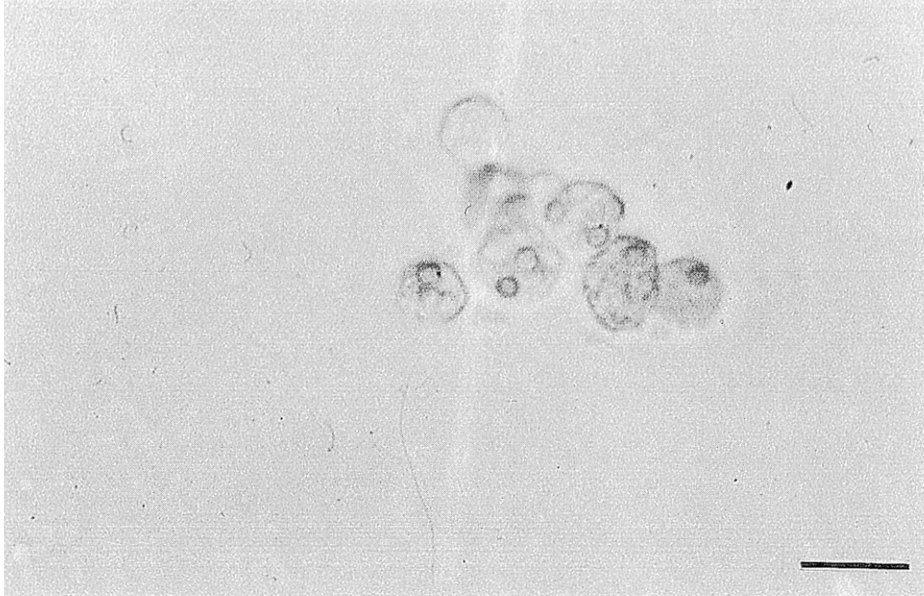

Fig. 9. SRBC phagocytosis by test cells. A-THP-1 cells were incubated with nonsensitized SRBC.

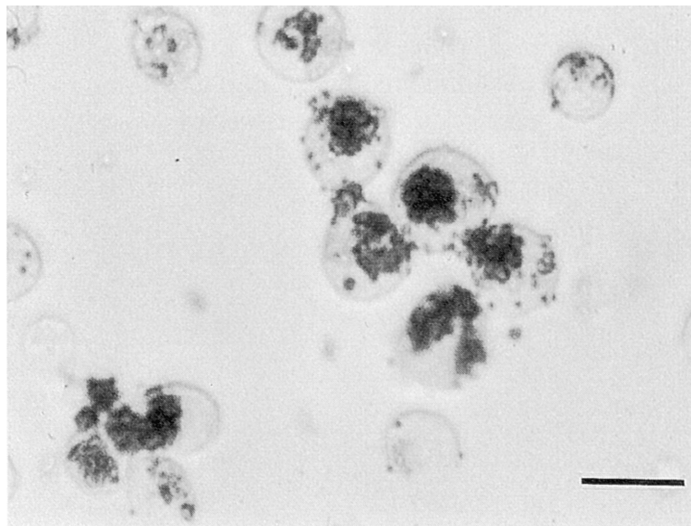

Fig. 10. Latex particle (Sekisui Chemical, 1.0 μm) phagocytosis of A-THP-1 cells in the absence of human serum. Remarkable phagocytic activity was demonstrated. Scale bar, 20 μm.

the other two cases (Table 5). There was no significant difference between O- and R-THP-1 cells.

Phagocytosis

Latex and SRBC phagocytosis of A-THP-1 cells was much elevated as compared to O- and R-THP-1 cells (Table 6, Figs. 9 and 10). The latex phagocytosis rate of A-THP-1 cells was almost 100%, while those of O- and R-THP-1 cells were both about 50%. Latex particle number ingested into single THP-1 cells also differed, being larger in the A-THP-1 (Fig. 10). SRBC

phagocytosis in the presence of heat-inactivated serum was not detected in O- and R-THP-1 cells. However, it was noted in 8% of A-THP-1 cells. In the presence of fresh human serum, SRBC phagocytosis of A-THP-1 cells increased to 27%, suggesting participation of complement receptors, but in O- and R-THP-1 cells this was not demonstrated (Table 6).

Fcγ receptor expression

Flow cytometric analysis was carried out for Fcγ receptor expression, using anti-CD64, anti-CD32 and anti-CD16 monoclonal antibodies (Table 7). O-THP-1 and R-THP-1 expressed FcγRI, but to a lesser extent than A-THP-1. In contrast FcγRII expression by A-THP-1 was relatively weak as compared to the other two THP-1 lines. None of the THP-1 cell lines expressed FcγRIII at all.

Expression of HLA-DR

HLA-DR expression was examined by flow cytometry (Table 8) and noted on more than 60% of A-THP-1 cells. O-THP-1 and R-THP-1 demonstrated much weaker HLA-DR expression.

TABLE 7. *Fcγ receptor expression on THP-1 cells*

	A-THP-1 (%)	O-THP-1 (%)	R-THP-1 (%)
Fcγ RI (CD64)	53	29	35
Fcγ RII (CD32)	61	84	84
Fcγ RIII (CD16)	0	0	0

FcγRI, FcγRII, FcγRIII on THP-1 cells were examined using anti-CD64, anti-CD32 and anti-CD16 specific monoclonal antibodies, respectively. FITC conjugated anti-mouse Ig diluted (1 : 20) with PBS plus 10% heat-inactivated normal human serum was used as a second antibody. Percentages of FcγR positive cells were measured by flow cytometry.

TABLE 8. *HLA-DR expression*

	A-THP-1 (%)	O-THP-1 (%)	R-THP-1 (%)
Exp. 1	81	15	9
Exp. 2	64	11	6
Exp. 3	68	NT	4

HLA-A-DR expression of THP-1 cells examined by flow cytometry using OKIa 1 antibody as the first antibody, and FITC-conjugated goat anti-mouse Ig diluted with PBS plus 10% heat inactivated normal human serum as a second antibody.

NT, not tested.

CD1a expression

The three THP-1 cell lines did not express CD1a (a dendritic cell maker).

Mixed lymphocyte reaction (MLR)

Since expression of HLA-DR differed significantly among the cell lines, MLR was performed in order to examine stimulator activity. In this test, allogeneic T cells served as responder cells. In order to exclude the possibility of antigen presentation by autologous B cells, T cells were purified with a T cell recovery column (Funakoshi, Tokyo). The three THP-1 cells all demonstrated stimulator activity (Fig. 11), but this was strongest with A-THP-1. The stimulation index (SI) of A-THP-1 was 7.47, when 1×10^5 A-THP-1 cells were used as stimulator, and significantly higher than respective values for O-THP-1 and R-THP-1 ($p<0.01\%$). These results indicated HLA-DR expression correlated well with MLR stimulator activity.

Complement receptor expression

Complement receptor (CR) expression was examined by flow cytometry using Mac 1 antibody (anti-CR3, iC3b receptor) and anti-CR4 (receptors for p150. 90, CD11c) (Table 9). Complement receptor expression of O-THP-1 and R-THP-1

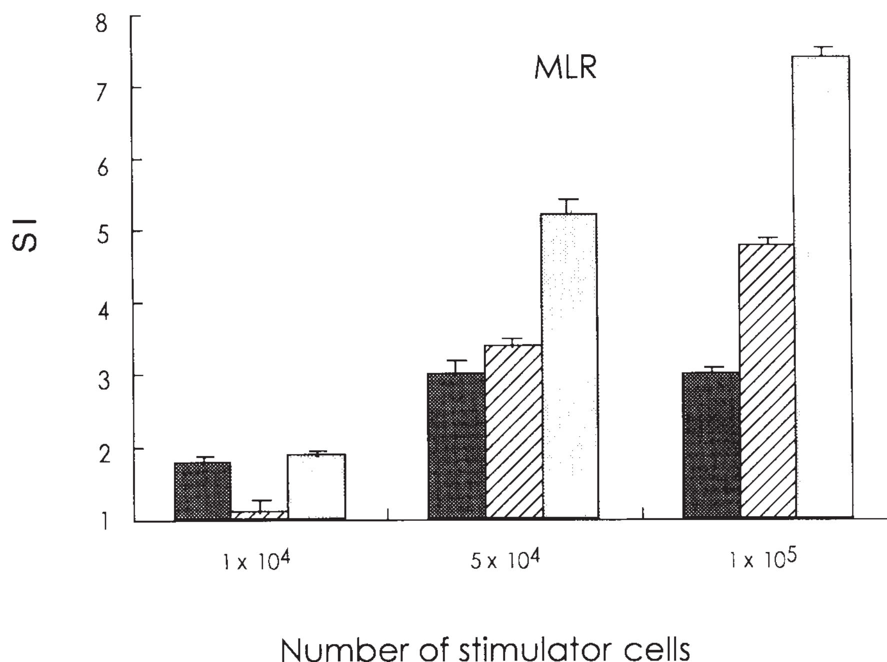

Fig. 11. Mixed lymphocyte reaction (MLR) test. Aliquots of MMC-treated (50 μg/ml at 37°C) THP-1 cells (as stimulator) and 4×10^6 allogeneic peripheral blood T cells (enriched by T-cell recovery column, Funakoshi, Tokyo) obtained from a healthy donor as responder cells were cocultured for 4 days, and then ^{3}H-thymidine uptake (24 hours pulse) was examined. 1 stimulator index (SI) is equivalent to 8189 cpm.
■, O-THP-1; ▨, R-THP-1; ▒, A-THP-1.

was weak, but both CR3 and CR4 were remarkably enhanced on A-THP-1 cells. These results coincided well with the results of SRBC rosette test in the presence of fresh serum (Table 6).

Effect of cytokines on THP-1 cells

Various cytokines, such as γ-INF, TNF, IL-4, and GM-CSF, are well known activators of monocytes. Morphological changes were examined on addition of IL-1α, IL-3, IL-4, IL-6, GM-CSF, α-tumor necrosis factor (α-TNF), γ-IFN to cultures of the three THP-1 cell lines (Table 10). Cell adherence of O-THP-1 and R-THP-1 cells increased 24 hours after addition of γ-IFN, but processes and multinucleated giant cells like A-THP-1 were not found. Processes of A-THP-1 cells became more prominent on addition of γ-IFN.

HLA-DR expression of O-THP-1 and R-THP-1 cells was enhanced by γ-IFN treatment to the levels almost equivalent to that of nontreated A-THP-1 cells (Table 10). That of A-THP-1 cells was further enhanced by addition of γ-IFN. Expression of FcγRI by the three THP-1 cell lines was increased (Table 11), but

TABLE 9. *C3 receptor expression of THP-1 cells examined by flow cytometry*

	A-THP-1 (%)	O-THP-1 (%)	R-THP-1 (%)
CR3 (Mac-1)	61	10	14
CR4 (CD11c)	73	4	25

For detection of CR3, TIB 128 culture supernatant was used as the first antibody. For CR4 detection, the FK24 antibody was applied.

TABLE 10. *Effects of various cytokines on HLA-DR expression of THP-1 cells*

Cytokines added to	A-THP-1 (%)	R-THP-1 (%)
No cytokine	68	4
γ-INF 100 U/ml	82	52
γ-INF 200 U/ml	91	59
α-TNF 50 U/ml	77	5
α-TNF 100 U/ml	85	9
IL-4 100 U/ml	83	3
IL-6 100 U/ml	70	9

Aliquots of cells (1×10^6) incubated with the indicated cytokines for 3 days and recovered cells were examined for HLA-DR expression by flow cytometry using the OKIa 1 antibody.

TABLE 11. *Effects of γ-INF on Fcγ receptor expression*

Expression of	FcγRI (CD64)		FcγRII (CD32)	
Induced by	Medium (%)	γ-IFN (%)	Medium (%)	γ-IFN (%)
A-THP-1	35	67	61	59
O-THP-1	29	53	84	84
R-THP-1	35	64	84	89

THP-1 cells (1×10^6) were incubated in the presence or absence of 100 U/ml γ-IFN for 4 days and recovered cells were examined for CD64 and CD32 expression by flow cytometry.

TABLE 12. *Secretion of IL-1β from THP-1 cells*

Cells	IL-1β concentration (pg/ml)
A-THP-1	76.7 ± 1.8
O-THP-1	12.3 ± 3.3
R-THP-1	21.5 ± 3.6
U937	<10

THP-1 cells (5×10^6) were cultured with 5 ml of culture medium for 3 days, then the recovered culture supernatant was examined for IL-1β with an IL-1β assay ELISA kit (Otsuka Pharmaceutical Inc., Tokushima).

that of FcγRII was not changed by γ-IFN. Other cytokines and monokines did not influence the morphology, adherence, or HLA-DR expression.

IL-1β assay

Secretion of IL-1β from A-THP-1 was remarkably increased compared with O-THP-1, R-THP-1 cells and U937 cells (Table 12).

Phenotype of cloned A-THP-1 cell line

A-THP-1 C1 cells showed a morphology essentially identical to the parent A-THP-1 cells, with increased phagocytic activity and elevated HLA-DR expression. A-THP-1 C1 cells were cultivable with RPMI 1640 supplemented with 10% FBS using 25 cm^2 flask. Subculture was usually performed twice a week, changing about 4 ml of cell suspension. Flasks for cultivation of A-THP-1 C1 were changed every 2 months. A-THP-1 C1 cells did not change characters even after freezing with the regular freezing solution made of 10% DMSO, 0.1% methylcellulose (cp 4000, Sigma Chemical Co., St. Louis, MO, USA) and RPMI-1640.

Discussion

In this study, we succeeded in establishing an activated macrophage cell line, A-THP-1. Usually, stimulation by LPS, phorbol ester, or cytokines is required to induce activation of macrophage cells. Activation of THP-1 cells by TPA (Tsuchiya et al. 1982), and γ-INF (Werner-Felmayer et al. 1990) has been reported. The A-THP-1 cell line, on the other hand, maintains an activated state without exogenous stimulation. Since we noted remarkable morphological differences between original THP-1 and A-THP-1, we have to exclude the possibility of contamination with third party cells. The results of nonspecific esterase staining, LDH isozyme pattern, chromosomal pattern, however, were identical to those for original THP-1 cells. In addition, DNA fingerprinting of A-THP-1 cell line by amplified fragment length polymorphism (AMP-FLP) using PCR with a D1S80 primer demonstrated essentially an identical pattern to the original THP-1 cell line. These data confirmed that the A-THP-1 cell line is derived from original THP-1 cell line.

Mycoplasma contamination sometime induces morphological change, and therefore two methods for its detection were carried out. Both gave negative results. In order to further exclude the possibility on effect of mycoplasma contamination, an anti-mycoplasma drug (MC210, Dainippon Pharmaceutical Inc., Osaka) was continuously added to culture of A-THP-1 cells, but the morphology and actions of A-THP-1 did not change at all.

A single clone of A-THP-1 C1, obtained by the limited dilution method, maintained the same morphology and character of A-THP-1 cells, suggesting that dividing cells are mononucleated, and adherent cells rapidly differentiate to form multinucleated, large cells. They are generated by cell fusion between actively dividing mononuclear cells during cultivation. It is well accepted that multinucleated macrophage cells can arise by cell fusion (Hassan et al. 1989). On transferring aliquots of cell suspensions consisting of mostly mononuclear A-THP-1 cells to new flasks, we found transferred nonadherent cells began to adhere to flask one hour after cell transfer and multinucleated cells then increased gradually, along with the nuclear number per cell. This observation also supports the conclusion that multinucleated cells are related to cell contact between mononucleated A-THP-1 cells.

The morphology of A-THP-1 cells resembled dendritic cells (Wood et al. 1985; Landry et al. 1988), and therefore we examined surface markers. A-THP-1 cells did not express CD1, though HLA-DR expression was remarkably increased. As A-THP-1 cells had FcγR, C3 receptors and phagocytotic activity, we can conclude that they belong to the macrophage rather than a dendritic lineage.

As activated macrophages, A-THP-1 cells demonstrated enhanced stimulator activity in the MLR test (Fig. 11) and enhanced production of IL-1β (Table 12), while TNF was not detected in the culture supernatant by L929 bioassay (data not

shown).

Complement receptor expression on A-THP-1 cells was found to be remarkably increased as indicated by flow cytometry examination and SRBC phagocytosis in the presence of fresh human serum, containing natural antibody to SRBC and complement (Table 6). Expression of CR3 and CR4 was elevated (Table 9). Four complement receptors (Myones et al. 1988), CR1, CR2, CR3, CR4, have been reported to be distributed in the monocyte series. It is thus of interest that the activated macrophage cell line A-THP-1 strongly expressed CR4, for there have been few reports of CR4 expression on macrophages.

Three classes of FcγR have selectivity for IgG isotypes (Klaassen et al. 1990). They have differing distributions (Deo et al. 1997). For example, monocytes/macrophages, peripheral blood monocytes and the U937 cell line express FcγRI and FcγRII (Looney et al. 1986), while cultivated peripheral blood monocytes demonstrate FcγRIII (Klaassen et al. 1990). Tissue macrophages generally express all three FcγRs (Anderson et al. 1990). In the present case the reason why FcγRIII was not detected (Table 7) remains unclear but abundant FcγRI was noted on A-THP-1.

In this study, examination of antibody dependent cell mediated cytotoxicity (ADCC) gave negative results for all three THP-1 cell lines (data not shown) in line with previous findings for the original THP-1 cells (Tsuchiya et al. 1980). ADCC activity and phagocytosis can not be discriminated since labeled target cells are ingested into phagocytic cells (Munn and Cheung 1990), as shown in the present study with EA rosetting by A-THP-1. Therefore, possibility of ADCC activity being masked by phagocytosis remains.

Addition of IL-4 to human peripheral blood monocytes induces appearance of long processes and enhanced expression of HLA-class II, CR3, CR4 (te Velde et al. 1988; Aiello et al. 1990), while it suppresses FcγR expression (te Velde et al. 1990). Mouse IL-4 causes cell fusion to bone marrow cells and alveolar macrophages (McInnes and Rennick 1988). GM-CSF elevates HLA-DR expression and secretion of IL-1 while γ-IFN enhances both HLA-DR and FcγR expression (Heidenreich et al. 1989). Based on these data, various cytokines were added to THP-1 cell lines in order to determine variation in their effects. Addition of γ-IFN caused enhanced expression of HLA-DR, FcγRI, and cell adherence in O-THP-1 and R-THP-1 cells (Tables 10 and 11). α-TNF enhanced HLA-DR expression of A-THP-1 cells slightly, but did not influence O-THP-1 and R-THP-1 cells (Table 10). Other cytokines did not affect expression of O-THP-1 and R-THP-1 cells. In order to ascertain whether unknown factors secreted from A-THP-1 cells might cause conversion of original THP-1 cells, transfer of conditioned culture medium from A-THP-1 was performed, but this did not cause any morphological changes. However, further experiments, such as combined administration of two or three kinds of cytokines, together with conditioned culture medium from A-THP-1 or longer duration cell treatment are necessary if this

possibility is to be excluded.

In conclusion, this is the first cell line to our knowledge which shows characteristics of activated macrophages without exogenous stimulation. Therefore A-THP-1 cells may serve as good materials for analysis of activation mechanisms of macrophages and of their multinucleation.

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References

1) Aiello, F.B., Longo, D.L., Overton, R., Takacs, L. & Durum, S.K. (1990) A role for cytokines in antigen presentation: IL-1 and IL-4 induce accessory functions of antigen-presenting cells. *J. Immunol.*, **144**, 2572-2581.
2) Anderson, C.L., Looney, R.J., Culp, D.J., Ryan, D.H., Fleit, H.B., Utell, M.J., Frampton, M.W., Manganiello, P.D. & Guyre, P.M. (1990) Alveolar and peritoneal macrophages bear three distinct classes of Fc receptors for IgG. *J. Immunol.*, **145**, 196-201.
3) Chen, T.R. (1977) In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. *Exp. Cell Res.*, **104**, 255-262.
4) Crawford, R.M., Finbloom, D.S., Ohara, J., Paul, W.E. & Meltzer, M.S. (1987) B cell stimulatory factor-1 (interleukin 4) activates macrophages for increased tumoricidal activity and expression of Ia antigens. *J. Immunol.*, **139**, 135-141.
5) Deo, Y.M., Granziano, R.F., Repp, R. & van de Winkel, J.G.J. (1997) Clinical significance of IgG Fc receptors and FcγR-directed immunotherapies. *Immunol. Today*, **18**, 127-135.
6) Geissler, K., Harrington, M., Strivastrava, C., Leemhuis, T., Tricot, G. & Broxmeyer, H.E. (1989) Effects of recombinant human colony stimulating factors (CSF) (Granulocyte-macrophage CSF, Granulocyte CSF and CSF-1) on human monocyte/macrophage differentiation. *J. Immunol.*, **143**, 140-146.
7) Grabstein, K.H., Urdal, D.L., Tushinski, R.J., Mochizuki, D.Y., Price, V.L., Cantrell, M.A., Gillis, S. & Conlon, P.J. (1986) Induction of macrophage tumoricidal activity by granulocyte-macrophage colony stimulating factor. *Science*, **232**, 506-508.
8) Hassan, N.F., Kamani, N., Meszaros, M.M. & Douglas, S.D. (1989) Induction of multinucleated giant cell formation from human blood-derived monocytes by phorbol myristate acetate in in vitro culture. *J. Immunol.*, **143**, 2179-2184.
9) Hay, R.J., Macy, M.L. & Chen, T.R. (1989) Mycoplasma infection of cultured cells. *Nature*, **339**, 487-488.
10) Heidenreich, S., Gong, J.H., Schmidt, A., Nain, M. & Gemsa, D. (1989) Macrophage activation by granulocyte/macrophage colony-stimulating factor. Priming for enhanced release of tumor necrosis factor-α and prostaglandin E_2. *J. Immunol.*, **143**, 1198-1205.
11) Hoessly, M.C., Rossi, R.M. & Fischkoff, S.A. (1989) Factors responsible for variable reported lineages of HL-60 cells induced to mature with butyric acid. *Cancer Res.*, **49**, 3594-3597.
12) Hori, K., Mihich, E. & Ehrke, M.J. (1989) Role of tumor necrosis factor and interleukin 1 in γ-interferon-promoted activation of mouse tumoricidal macrophages. *Cancer Res.*, **49**, 2606-2614.
13) Hudson, M.M., Snyder, J.S., Jaffe, N. & Kleinerman, E.S. (1988) In vitro and in vivo

effects of adriamycin therapy on monocyte activation by liposome-encapsulated immunomodulators. *Cancer Res.*, **48**, 5256–5263.

14) Imai, Y., Yamakawa, M., Sato, T. & Suda, A. (1989) Malignant fibrous histiocytoma: Similarities to the "fibrohistiocytoid cells" in chronic inflammation. *Virchow Archiv-A, Pathological Anatomy & Histopathology*, **414**, 285–298.
15) Klaassen, R.J., Ouwehand, W.H., Huiznga, T.W., Engelfriet, C.P. & von dem Borne, A.E. (1990) The Fc-receptor III of cultured human monocytes. Structural similarity with FcRIII of natural killer cells and role in the extracellular lysis of sensitized erythrocytes. *J. Immunol.*, **144**, 599–606.
16) Kornbluth, R.S. & Edington, T.S. (1986) Tumor necrosis factor production by human monocytes is a regulated event: Induction of TNFα mediated cellular cytotoxicity by endotoxin. *J. Immunol.*, **137**, 2585–2591.
17) Kudo, T. & Tachibana, T. (1984a) Phenotype characteristics of human B cells studied by Epstein-Barr virus infection. II. C3 receptor switching during human B cell differentiation. *Immunol. Lett.*, **6**, 343-348.
18) Kudo, T. & Tachibana, T. (1984b) Phenotype characteristics of human B cells studied by Epstein-Barr virus infection. III. In vitro modulation of C3 receptor expression on human lymphoid cells. *Immunol. Lett.*, **8**, 349-353.
19) Landry, D., Lafontaine, M., Cossette, M., Barthelemy, H., Chartrand, C., Montplaisir, S. & Pelletier, M. (1988) Human thymic dendritic cells. Characterization, isolation and functional assays. *Immunology*, **65**, 135–142.
20) Looney, R.J., Abraham, G.N. & Anderson, C.L. (1986) Human monocytes and U937 cells bear two distinct Fc receptors for IgG. *J. Immunol.*, **136**, 1641–1647.
21) McInnes, A. & Rennick, D.M. (1988) Interleukin 4 induces cultured monocytes/macrophages to form giant multinucleated cells. *J. Exp. Med.*, **167**, 598–661.
22) Munn, D.H. & Cheung, N.K. (1989) Antibody-dependent antitumor cytotoxicity by human monocytes cultured with recombinant macrophage colony-stimulating factor. Induction of efficient antibody-mediated antitumor cytotoxicity not detected by isotope release assays. *J. Exp. Med.*, **170**, 511-526.
23) Munn, D.H. & Cheung, N.K. (1990) Phagocytosis of tumor cells by human monocytes cultured in recombinant macrophage colony stimulating factor. *J. Exp. Med.*, **172**, 231–237.
24) Myones, B.L., Daizell, J.G., Hogg, N. & Ross, G.D. (1988) Neutrophil and monocyte cell surface p150, 95 has iC3b-receptor (CR4) activity resembling CR3. *J. Clin. Invest.*, **82**, 640–651.
25) Najar, H.M., Ruhl, S., Bru-Capdeville, A.C. & Peters, J.H. (1990) Adenosine and its derivatives control human monocyte differentiation into highly accessory cells versus macrophages. *J. Leukoc. Biol.*, **47**, 427-439.
26) Nasiell, M., Roger, V., Nasiell, K., Enstad, I., Vogel, B. & Bisther, A. (1972) Cytologic findings indicating pulmonary tuberculosis. I. The diagnostic significance of epitheloid cells and Langhans' giant cells found in sputum or bronchial secretions. *Acta Cytol.*, **16**, 146–151.
27) Numasaki, M., Fukuoka, Y., Kudo, T., Saeki, H., Tachibana, T., Motomiya, M. & Nukiwa, T. (1995) A novel human monoclonal antibody, TONO-1, reactive with T-lymphocytic leukemia cells. *Int. J. Cancer*, **62**, 42–47.
28) Santamaria, P., Gehrz, R.C., Bryan, M.K. & Barbosa, J.J. (1989) Involvement of class II MHC molecules in the LPS-induction of IL-1/TNF secretions by human monocytes. Quantitative differences at the polymorphic level. *J. Immunol.*, **143**, 913–922.
29) Smith, P.D., Lamerson, C.L., Wong, H.L., Wahl, L.M. & Wahl, S.M. (1990) Granulocyte-macrophage colony-stimulating factor stimulates human monocyte accessory cell function. *J. Immunol.*, **144**, 3829-3834.
30) Stuart, P.M., Zlotnik, A. & Woodward, J.G. (1988) Induction of class I and class II

MHC antigen expression on murine bone marrow-derived macrophages by IL-4 (B cell stimulatory factor 1). *J. Immunol.*, **140**, 1542-1547.

31) te Velde, A.A., Klomp, J.P., Yard, B.A., de Vries, J.E. & Figdor, C.G. (1988) Modulation of phenotypic and functional properties of human peripheral blood monocytes by IL-4. *J. Immunol.*, **140**, 1548-1554.

32) te Velde, A.A., Huijbens, R.J., de Vries, J.E. & Figdor, C.G. (1990) IL-4 decreases FcγR membrane expression and FcγR-mediated cytotoxic activity of human monocytes. *J. Immunol.*, **144**, 3046-3051.

33) Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T. & Tada, K. (1980) Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int. J. Cancer*, **26**, 171-176.

34) Tsuchiya, S., Kobayashi, Y., Goto, Y., Okuyama, H., Nakae, S., Konno, T. & Tada, K. (1982) Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester. *Cancer Res.*, **42**, 1530-1536.

35) Weinberg, J.B., Hobbs, M.M. & Misukonis, M.A. (1984) Recombinant human γ-interferon induces human monocyte polykaryon formation. *Proc. Natl. Acad. Sci. USA*, **81**, 4554-4557.

36) Werner-Felmayer, G., Werner, E.R., Fuchs, D., Hausen, A., Reibnegger, G. & Wachter, H. (1990) Neopterin formation and tryptophan degradation by a human myelomonocytic cell line (THP-1) upon cytokine treatment. *Cancer Res.*, **50**, 2863-2867.

37) Wood, G.S., Turner, R.R., Shiurba, R.A., Eng, L. & Warnke, R.A. (1985) Human dendritic cells and macrophages: In situ immunophenotypic definition of subsets that exhibit specific morphologic and microenvironmental characteristics. *Am. J. Pathol.*, **119**, 73-82.